REMARKS

Claims 146, 148-151, and 159-162 are pending. Claims 146, 148-151, and 159-162 are rejected under 35 U.S.C. § 102(b) for anticipation by both Furcht et al. (U.S. Patent No. 7,015,037; hereinafter "Furcht") and Yilmaz (U.S. Patent No. 7,510,877; hereinafter "Yilmaz"). By this reply, Applicant cancels claim 151, amends claims 146, 149, 159, and 160, and addresses each of the rejections.

Support for the Amendment

Support for the amendment to claims 146 and 149 is found in prior claim 151 and in the specification at, e.g., page 24, lines 16-23, page 42, lines 13-19, and page 64, line 10, through page 65, line 3. Claims 159 and 160 are amended to correct claim dependencies and for consistency. No new matter is added by the amendment.

Interview with Examiner Belyavskyi

The inventor, Dr. Denise Faustman, and the Applicant's representative, Dr. Todd Armstrong, wish to thank Examiner Belyavskyi for the courtesy of an in-person interview (the "Interview") on February 10, 2011. The novelty rejections over Furcht and Yilmaz were discussed and Applicant agreed to provide a Declaration from Dr. Faustman stating that the cells described in these publications are structurally and functionally different from the cell of present claims 146, 148-150, and 159-162. Applicant also agreed to amend independent claim 146 to recite that the Hox11+, CD45- cells are obtained from spleen or mobilized into the peripheral blood from the spleen.

The present claim amendments and the remarks below reflect the content of the interview.

Rejections under 35 U.S.C. § 102(b)

Furcht Fails to Anticipate the Cell of Present Claims 146, 148-150, and 159-162

Claims 146, 148-151, and 159-162 are rejected under 35 U.S.C. § 102(b) for anticipation by Furcht. As was discussed during the Interview, Furcht's MASCs are not the Hox11+, CD45- cells of present claims 146, 148-150, and 159-162.

Anticipation requires that "each element of the claim at issue is found, either expressly described or under the principles of inherency, in a single prior art reference or that the claimed invention was previously known or embodied in a single prior art device or practice." *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760, 771 (Fed. Cir. 1983); *see also In re Samour*, 571 F.2d 559, 562 (CCPA 1978) (the key question is whether a single prior art reference "discloses every material element of the claimed subject matter"). To the extent that the Office's anticipation rejection rests on inherency, M.P.E.P. § 2112(IV) summarizes the legal standard with respect to the requirements for a rejection based on inherency. According to the M.P.E.P., the Office must first provide the rationale or evidence tending to show inherency. The standard, cited below, requires that the missing descriptive matter is *necessarily present* in the thing described in the reference and that it would be so recognized by persons of ordinary skill.

> "To establish inherency, the extrinsic evidence 'must make clear that *the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill.* Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'" *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citations omitted) (The claims were drawn to a disposable diaper having three fastening elements. The reference disclosed two fastening elements that could perform the same function as the three fastening elements in the claims. The court construed the claims to require three separate elements and held that the reference did not disclose a separate third fastening element, either expressly or inherently.).

(M.P.E.P. § 2112(IV); emphasis added.) Moreover, as cited below, the Federal Circuit has made clear that disclosure of a broad genus does not inherently disclose all species within a category and that an invitation to investigate is not equivalent to an inherent disclosure.

> Also, "[a]n invitation to investigate is not an inherent disclosure" where a prior art reference "discloses no more than a broad genus of potential applications of its discoveries." *Metabolite Labs., Inc. v. Lab. Corp. of Am. Holdings*, 370 F.3d 1354, 1367, 71 USPQ2d 1081, 1091 (Fed. Cir. 2004) (explaining that "[a] prior art reference that discloses a genus still does not inherently disclose all species within that broad category" but must be examined to see if a disclosure of the claimed species has been made or whether the prior art reference merely invites further experimentation to find the species.

(M.P.E.P. § 2112(IV).)

As was discussed during the Interview, Furcht fails to teach the isolation of Hox11, CD45- cells from spleen or from the peripheral blood following mobilization from the spleen. Furcht describes only multipotent adult stem cells (MASCs) isolated from bone marrow (Example 1, col. 44) and mentions brain, liver, and "possibly other organs" as sources for MASCs (col. 14, lines 50-59), yet Furcht fails to establish that its MASCs are Hox11+, CD45- cells, nor does Furcht recognize that such cells can be isolated from spleen or mobilized into peripheral blood from the spleen . Thus, Furcht clearly fails to teach Hox11+, CD45- spleen cells or that its MASCs are *necessarily* Hox11+, CD45- and would be so recognized by persons of ordinary skill, as is required (M.P.E.P. § 2112(IV)).

As evidence that Furcht's MASCs are not Hox11+, CD45- cells, Applicant directs the Examiner to the Declaration of Dr. Denise Faustman (the "Declaration"), provided herewith, which states that an examination of tissue from bone marrow, kidney, liver, and tonsil shows that Hox11 expression is *completely absent* in these tissues (See ¶ 4 of the Declaration and Exhibit 1). Furthermore, as evidenced by Watt et al. (Gene 323:89-99, 2003; a copy of the abstract is provided), brain tissue appears to lack expression of Hox11, as well. Thus, none of the tissues described by Furcht as a source (or potential source) for MASCs, in particular bone marrow, brain, and liver, exhibit Hox11 expression. Accordingly, Furcht's MASCs are not Hox11 expressing cells and Furcht fails to inherently anticipate present claims 146, 148-150, and 159-162.[1]

Finally, Applicant notes that Furcht only describes Hox11 expression in chondrocytes and osteocytes that were differentiated from MASCs (see Table 2 at col. 20). Chondrocytes and osteocytes lack the ability to differentiate into two or more different cells types, as is required by present claim 161. In addition, *Hox11*-expressing cells were previously not known to be found in adult spleen (see, e.g., page 2913 of Dear et al., *Development* 121:2909-2915, 1995; "*Hox11* expression continues in the normal spleen up to at least E18.5 and no expression is found in adult

[1] Even if Furcht had demonstrated the presence of Hox11+, CD45- cells, which it does not do, Furcht is not an enabling anticipatory reference because it fails to teach the isolation of MASCs from any source other than bone marrow, and certainly not the spleen or peripheral blood (To be properly considered as being anticipatory, a prior art reference must contain an enabling disclosure. *Chester v. Miller*, 906 F.2d at 1576 n.2, 15 U.S.P.Q.2d at 1336 n.2 (Fed. Cir. 1990); *see also Titanium Metals Corp. of America v. Banner*, 778 F.2d at 781, 227 U.S.P.Q. at 778 (Fed. Cir. 1985); *Scripps Clinic & Research Found. v. Genentech, Inc.*, 927 F.2d 1565, 1578, 18 U.S.P.Q.2d 1001, 1011 (Fed. Cir. 1991); *Helifix Ltd. v. Blok-Lok, Ltd.*, 208 F.3d 1339, 54 U.S.P.Q.2d 1299 (Fed. Cir. 2000), citing *In re Donohue*, 766 F.2d 531, 533, 226 U.S.P.Q. 619, 621 (Fed. Cir. 1985)).

spleen"; a copy is provided herewith). For this reason, as well, Furcht fails to teach the Hox11, CD45- cells of present independent claim 146, and claims dependent therefrom. This rejection should be withdrawn.

Yilmaz Fails to Anticipate the Cell of Present Claims 146, 148-150, and 159-162

The Office also rejects claims 146, 148-150, and 159-162 for anticipation by Yilmaz. The Office states that Yilmaz:

> teaches...isolated mammalian cells that are adult spleen cells. (see entire document. Claim 13 in particular).
> As is evidenced from the instant specification, said cells are inherently endogenously expressing HOX 11 and CD45 negative (see paragraph 88 and 0115 in particular)

(Office Action, p. 3.)

As was discussed during the Interview, Yilmaz describes only hematopoietic stem cells (HSCs) that express CD150+ and lack expression of CD48 and CD244 (Abstract). HSCs are understood in the art to be CD45+ cells, and thus Yilmaz's cells are not the same as the CD45- cells of present independent claim 146, and claims dependent therefrom. As evidence, Applicant directs the Office to Table 2 of Wilson and Trumpp (*Nat. Rev. Immunol.* 6:93-106, 2006; a copy is provided herewith), which states that CD150+, CD48- HSCs mobilized from the spleen are CD45+. Thus, Yilmaz does not explicitly or inherently describe the isolation of any Hox11+, CD45- cells (M.P.E.P. § 2112(IV), *supra*).

As further evidence, Applicant directs the Office to ¶ 5 of the Declaration, which states that, prior to the present application, CD45+ splenocytes were known in the art and constitute the majority of cells in the spleen. Dr. Faustman states that the presence of a Hox11+, CD45- cell in the spleen was not previously recognized (see Dear et al., *supra*), and thus one of skill in the art would not necessarily have recognized that this cell species was even present in the spleen or could be mobilized from the spleen based on Yilmaz. Yilmaz fails to teach the Hox11+, CD45- cells of present independent claim 146, and claims dependent therefrom, and thus this rejection should also be withdrawn.

CONCLUSION

Applicant submits that present claims 146, 148-150, and 159-162 are in condition for allowance, and such action is respectfully requested.

Also transmitted herewith is a petition to extend the period of time for response to the Office Action by one month, to and including April 14, 2011.

If there are any other charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 14 April 2011

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Gene. 2003 Dec 24;323:89-99.

Specific alternative HOX11 transcripts are expressed in paediatric neural tumours and T-cell acute lymphoblastic leukaemia.

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Abstract

HOX11 is a proto-oncogene, which is silent in normal mature T-cells, while being aberrantly activated in T-cell acute lymphoblastic leukaemia (T-ALL) by translocations t(10;14)(q24;q11) or t(7;10)(q35;q24). Although many oncogenes are expressed in alternative forms in cancer, thus far, only one form of the human HOX11 transcript has been reported. We describe here the identification of three alternative transcripts of the HOX11 proto-oncogene, expressed in primary T-ALL specimens. Using rapid amplification of cDNA ends (RACE) and targeted RT-PCR, we have sequenced 23 individual cDNA clones characterising these novel transcripts. Northern hybridisation identified particular novel exons expressed in T-ALL, which are not expressed in normal T-cells. To date, aberrant expression of HOX11 has only been associated with leukaemia. Our survey of a range of neuroblastoma and primitive neuroectodermal tumour (PNET) cell lines demonstrated the expression of these novel HOX11 transcripts in tumours of neural origin, while their expression was not detected in normal brain tissues. Strikingly, the dominant transcript in these neural tumour cell lines is more than 1 kb larger than the dominant transcript in T-ALL. These observations, combined with sequence data from several EST clones derived from medulloblastoma cDNA libraries, support a new hypothesis that HOX11 may also function as a neural oncogene or brain tumour marker.

PMID: 14659882 [PubMed - indexed for MEDLINE]

REVIEWS

Bone-marrow haematopoietic-stem-cell niches

Anne Wilson and Andreas Trumpp‡*

Abstract | Adult stem cells hold many promises for future clinical applications and regenerative medicine. The haematopoietic stem cell (HSC) is the best-characterized somatic stem cell so far, but *in vitro* expansion has been unsuccessful, limiting the future therapeutic potential of these cells. Here we review recent progress in characterizing the composition of the HSC bone-marrow microenvironment, known as the HSC niche. During homeostasis, HSCs, and therefore putative bone-marrow HSC niches, are located near bone surfaces or are associated with the sinusoidal endothelium. The molecular crosstalk between HSCs and the cellular constituents of these niches is thought to control the balance between HSC self-renewal and differentiation, indicating that future successful expansion of HSCs for therapeutic use will require three-dimensional reconstruction of a stem-cell–niche unit.

Self-renewal
The capacity of a stem cell to divide in such a way that one or both daughter cells retain the stem-cell fate.

Steel-Dickie mice
(Sl/Sl^d). A spontaneous mouse mutant with a defect in the production of membrane-bound stem-cell factor (SCF), although secreted SCF is produced normally.

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Adult stem cells are present in most self-renewing tissues, including the skin, the intestinal epithelium and the haematopoietic system. On a single-cell basis, they have the capacity both to produce more stem cells of the same type (that is, to self-renew) and to give rise to a defined set of mature differentiated progeny to maintain or repair their host tissue[1–3]. The best-characterized adult stem cell is the haematopoietic stem cell (HSC)[4,5]. Since HSCs were first identified[6], advances in technology have made it possible to purify adult mouse HSCs close to homogeneity. Several groups have achieved long-term reconstitution of the haematopoietic system of a lethally irradiated mouse by transplantation of a single purified bone-marrow HSC, providing functional proof of the existence of adult HSCs[2,7–9]. Maintenance of HSCs and regulation of their self-renewal and differentiation *in vivo* is thought to depend on their specific microenvironment, which has been historically called the haematopoietic-inductive microenvironment[10] or 'stem-cell niche'[11]. The crucial role of the microenvironment for HSC function has long been recognized because a mutation in the gene encoding membrane-bound stem-cell factor (SCF, also known as KIT ligand) that is present in *Sl/Sl^d* mice (steel-Dickie mice) causes changes in the HSC niche and leads to the failure of bone-marrow HSC maintenance *in vivo*[12–14]. Nevertheless, the structure and localization, as well as the molecular and cellular basis for niche activity, have long remained a 'black box'. It is only recently that the concept of a stem-cell niche has been supported by data on the molecules and cell types that are involved in its formation, first in invertebrates and more recently in mammals[4,15–17]. Many of the different types of signals that are exchanged between stem cells and niche cells, as well as some of the signalling pathways that control stem-cell maintenance, self-renewal and differentiation, have recently been identified. In this Review, we discuss models for the different types of bone-marrow HSC niches that might exist, particularly focusing on the molecules that are known to coordinate HSC function *in vivo*.

The adult HSC

Murine HSCs were initially identified on the basis of their ability to form colonies in the spleens of lethally irradiated mice following bone-marrow transfer[6,18]. Subsequently, a number of assays have been developed to monitor HSC activity *in vivo* and *in vitro* (BOX 1). The most widely accepted assay is the capacity of HSCs to provide lifelong reconstitution of all blood-cell lineages after transplantation into lethally irradiated recipients. The strictest version of this long-term repopulating (LTR) assay, known as serial transplantation, requires that HSC-containing donor bone marrow can be re-transplanted into secondary, and even tertiary, recipients while retaining both self-renewal and multi-lineage differentiation capacity[19]. These functional assays have been used to establish the cell-surface phenotype of mouse HSCs, allowing their prospective isolation by fluorescence-activated cell sorting (FACS) (BOX 1).

All functional HSCs are found in the population of bone-marrow cells that does not express the cell-surface

Box 1 | Characteristics of haematopoietic stem cells

Haematopoietic stem cells (HSCs) are defined functionally by their ability to mediate long-term repopulation of all blood-cell lineages (known as long-term repopulating (LTR) activity) and to form colony forming units in the spleen after transfer to lethally irradiated recipients. Assays to assess HSC activity *in vitro* include LTC-IC (long-term culture-initiating cell) and CAFC (cobblestone area-forming cell) assays[101].

All LTR HSCs are contained in the lineage-negative (Lin⁻) stem-cell antigen 1 (SCA1)⁺KIT⁺ (LSK) subset that comprises ~0.5% of bone marrow[102]. 100 LSK cells are sufficient for multi-lineage LTR activity[6]. Additional markers to further subdivide the LSK population into long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs), which have limited self-renewal activity, have been identified and are summarized in the figure. LTR activity is also enriched in the population of bone-marrow cells with low-level staining of rhodamine 123 (Rho)[103]. In addition, functional adult LTR HSCs can also be isolated by their ability to actively efflux the DNA-binding dye Hoechst 33342. This characteristic is designated as side-population (SP) ability[104,105].

Single-cell reconstitution studies have indicated the following frequencies for multi-lineage reconstitution and long-term engraftment:

- LSKThy1.1^low cells (18%)[7]
- SP⁺Rho^low Lin⁻ cells (40%)[106]
- LSKCD150⁺CD48⁻CD41⁻ cells (47%)[9]
- LSKSP⁺CD34⁻ cells (35%)[107] and (86%)[8]

LT-HSCs divide infrequently because (by DNA content) only ~5% are in the S or G_2/M phases of the cell cycle[11,108], and 60–70% of LSK cells are shown to be in G_0 by Ki67 staining[92]. Studies using bromodeoxyuridine (BrdU) uptake have calculated that LSK HSCs divide every 30–60 days[51,53,54]. 3.8% of LSK CD150⁺ HSCs are in the S or G_2/M phases of the cell cycle[9]. The low cycling status of HSCs might explain their significant resistance to cytotoxic drugs *in vivo*[40].

Label-retaining cells (LRCs) are defined by their capacity to retain the DNA label BrdU long-term (for 70 days). Lin⁻KIT⁺ LRCs are enriched for phenotypic HSCs, but due to the nature of the assay, functional LTR activity cannot be assessed.

LT-HSCs, ST-HSCs and haematopoietic progenitor cells show substantially different gene expression patterns[4,24–26].

FLT3, fms-related tyrosine kinase 3; MPP, multipotential progenitor; N-cad, N-cadherin; TIE2, tyrosine kinase receptor 2.

markers normally present on lineage (Lin)-committed haematopoietic cells but does express high levels of stem-cell antigen 1 (SCA1) and KIT. Therefore, this HSC-containing subset of bone-marrow cells is known as the LSK (Lin⁻SCA1⁺KIT⁺) subset. Because only some phenotypic LSK HSCs have LTR activity, they can be further subdivided into long-term (LT)-HSCs, which are CD34⁻ fms-related tyrosine kinase 3 (FLT3)⁻CD150⁺ and have LTR activity, and short-term (ST)-HSCs, which are CD34⁺FLT3⁻ and have only limited self-renewal activity[9,20–22] (BOX 1). Although it has been shown that 100 LSK HSCs can provide protection from lethal irradiation[23], several groups have succeeded in reconstituting all haematopoietic lineages from a single, purified HSC (BOX 1). These data clearly show that at the clonal-level HSCs fulfill the characteristics of true adult stem cells — multi-lineage reconstitution and long-term self-renewal. Recent gene-profiling studies have begun to establish a transcriptional signature of purified HSCs, which is the first step to elucidating the molecular mechanisms of HSC function[9,24–27]. Furthermore, the number of functional HSCs *in vivo* is altered in a large number of mutant mice (see Supplementary information S1 (table)), implicating several of these gene products in the regulation of self-renewal and differentiation of stem cells.

Asymmetric self-renewing division in stem cells

The vast majority of cell divisions are symmetrical, producing identical daughter cells and leading (in the absence of apoptosis) to increased numbers of cells. This process is readily observed for cells in culture and also occurs during organogenesis, where substantial cellular expansion (including stem cells) occurs during embryogenesis. By contrast, under homeostatic conditions in the adult, the number of tissue stem cells in a particular organ remains relatively constant, despite the fact that they proliferate, because they not only self-renew but also produce differentiated progeny.

This balance could be achieved if the number of stem cells dividing symmetrically to generate two identical daughter cells with stem-cell function was equivalent to the number of stem cells giving rise to two differentiated daughter cells. However, because this mechanism does not function at the single-cell level, and would require close coordination of two separate stem-cell populations, it is commonly assumed that an individual stem cell can give rise to two non-identical daughter cells, one maintaining stem-cell identity and the other becoming a differentiated cell. There are two mechanisms by which this asymmetry can be achieved, depending on whether it occurs pre- (divisional asymmetry), or post- (environmental asymmetry) cell division (FIG. 1).

Divisional asymmetry. In divisional asymmetry, specific cell-fate determinants in the cytoplasm (mRNA and/or proteins) redistribute unequally before the onset of cell division. During mitosis, the cleavage plane is oriented such that only one daughter cell receives the determinants. Therefore, two non-identical daughter cells are produced, one retaining the stem-cell fate while the other initiates differentiation (FIG. 1a).

In invertebrate model systems, the establishment of asymmetry by this mechanism is crucial for various developmental processes and the molecular basis for it has been well documented[28]. Asymmetrically localized proteins in *Drosophila melanogaster* include members of the partitioning defective (PAR) family of proteins, such as Insucteable (INSC) and Partner of Insucteable (PINS, the homologue of which is LGN in mammals), as well as NUMB, a negative modulator of Notch signalling[29]. However, only a few examples of divisional asymmetry have been documented in higher vertebrates[28,30]. For example, in the mammalian fetal epidermis, basal cells not only divide symmetrically to allow a two-dimensional expansion of the

epidermis, but also divide asymmetrically to promote stratification and differentiation of the skin. In this case, a protein complex that includes PAR3, LGN and a distant mouse homologue of *D. melanogaster* INSC (mINSC), forms an apical crescent that dictates the polarity of the ensuing cell division[30].

Although such a mechanism has not been shown in any vertebrate stem-cell type *in vivo*, a number of *in vitro* studies indicate that HSCs might undergo some type of asymmetric division. In an analysis of the ability of either of the two daughter cells derived from a single cultured HSC to long-term reconstitute lethally irradiated recipients, it was shown that ~20% of HSCs produced non-identical daughter cells[31–33]. However, these studies neither provide a mechanism for the observed asymmetry, nor show if it occurs pre- or post-cell-division. Moreover, whether these *in vitro* studies reflect the situation of bone-marrow HSCs remains unclear. Future studies will need to take advantage of recently developed tools to monitor asymmetric determinants such as mINSC and LGN[30] to determine whether, and to what extent, divisional asymmetry occurs in HSCs *in vitro*, and more importantly if it occurs in self-renewing HSCs in their niche.

Environmental asymmetry and the stem-cell niche concept. An alternative way to achieve asymmetry is by exposure of the two daughter stem cells to different extrinsic signals provided by distinct local microenvironments (FIG. 1b). Therefore, a stem cell would first undergo a symmetric self-renewing division, producing two identical daughter cells. While one daughter cell would remain in the niche microenvironment, conserving its stem-cell fate, the other would contact (passively or actively) a different microenvironment that would no longer preserve its stem-cell phenotype but would instead produce signals initiating differentiation[16,17]. Therefore, as with divisional asymmetry, the final product would be two non-identical daughter cells but achieved post-cell-division and not pre-cell-division (FIG. 1b).

Although the influence of the niche for stem-cell maintenance has been well documented, it has not been possible to monitor the division of vertebrate stem cells *in vivo*. However, recent studies of the mammalian epidermis indicate that the molecular mechanism for divisional asymmetry is conserved between invertebrates and vertebrates, raising the possibility that this mechanism might also mediate divisional asymmetry in mammalian stem cells (including HSCs). Therefore, it is possible that HSCs could undergo both divisional and environmental asymmetric divisions; therefore both mechanisms could be used in parallel by independent HSCs to direct non-stem-cell daughters to distinct cell fates.

Stem-cell-niche function

A stem-cell niche can be defined as a spatial structure in which stem cells are housed and maintained by allowing self-renewal in the absence of differentiation. Although the concept of the stem-cell niche was initially proposed in vertebrates[10,11], the *D. melanogaster* ovarian and testicular niches controlling germline stem-cell maintenance and differentiation were the first to be characterized[34,35].

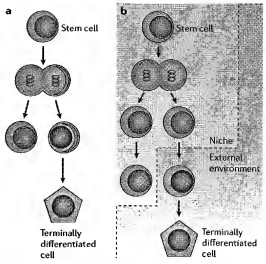

Figure 1 | A model of asymmetric cell division. **a** | During divisional asymmetry, cell-fate determinants are asymmetrically localized to only one of the two daughter cells, which retains stem-cell fate, while the second daughter cell differentiates. **b** | During environmental asymmetry, after division, one of two identical daughter cells remains in the self-renewing niche microenvironment while the other relocates outside the niche to a different, differentiation-promoting microenvironment.

In higher organisms, the analysis of stem-cell–niche interactions has been hampered by their unknown location. However, during the past few years, substantial progress has been made in localizing adult stem cells *in situ*. Many studies have indicated that most adult tissue stem cells (such as HSCs, or epidermal stem cells (ESCs) in the skin) divide infrequently and can be quiescent for weeks or even months[36–39]. In support of this notion, the adult-stem-cell pool is largely resistant to classical chemotherapeutic agents that target cycling cells[40]. In addition, HSCs that efficiently engraft after transplantation are mainly quiescent[41,42] and considered to be metabolically inactive[43]. Moreover, when the DNA of adult stem cells is labelled during cellular proliferation by nucleotide analogues (such as ^{3}H-thymidine or bromodeoxyuridine (BrdU labelling)), or by the histone H2B–enhanced-green-fluorescent-protein fusion protein (H2B–EGFP), the DNA label can be retained for months and has consequently been used to locate quiescent stem cells *in situ*[37–39,44,45]. For example, such label-retaining cells (LRCs) were initially identified using BrdU in the hair-follicle bulge in the skin, leading to the suggestion that ESCs were present in this structure[37,45]. However, the nature of this assay precludes a functional assessment of stem-cell activity post-identification, because to identify $BrdU^+$ cells, the cells must be fixed. Subsequently, H2B–EGFP was used to show that LRCs in the bulge are indeed functional ESCs[38].

In the bone marrow, only $BrdU^+$ LRCs have been identified in trabecular bone[39,45]. Nevertheless, in analogy to ESCs, $BrdU^+$ LRCs in the bone marrow are probably highly enriched for functional HSCs, particularly if they also fail to express differentiation markers, although this

BrdU labelling
Incorporation of bromodeoxyuridine (BrdU) into newly synthesized DNA permits indirect detection of proliferating cells using fluorescently labelled BrdU-specific antibodies by either flow cytometry or fluorescence microscopy

Trabecular bone
Also known as cancellous bone, this is found in areas of rapid turnover such as the ends of the long bones.

remains to be shown definitively. If so, these long-term quiescent HSCs are unlikely to contribute substantially to the normal homeostasis of the haematopoietic system with its high turnover rate. Instead, they might serve as a reserve pool that can be reactivated in response to stress or injury and might even be stored in a separate 'quiescent-storage' niche[16] (FIG. 2a). In response to myeloablative agents, HSCs are released into the circulation (a process known as mobilization, as discussed later), enter the cell cycle to re-establish haematopoiesis and migrate to putative HSC niches in the spleen and liver. After repair, they return to their bone-marrow niches and become quiescent again[27,47,48].

Although most adult stem cells are considered to be quiescent, this is not a requirement for all stem cells. For example, embryonic stem cells have enormous proliferative potential but retain their stem-cell fate, and fetal-liver HSCs, although highly proliferative, very efficiently reconstitute irradiated adult hosts[49,50]. Therefore, cell-cycle status might only reflect differences between fetal (that is, clonally expanding) HSCs and adult (that is, steady-state) HSCs rather than be a measure of stem-cell fate. Moreover, even during homeostasis, a proportion of stem cells are expected to divide at least occasionally (particularly in highly regenerative tissues such as the haematopoietic system), to maintain a constant flow of short-lived progenitors that can generate enough cells to replace those that are constantly lost during normal turnover. Indeed, continuous BrdU labelling has revealed a considerable number of cycling HSCs[51,52].

It is currently unclear whether all postulated stem-cell-niche functions (storage of quiescent stem cells, self-renewal and inhibition of differentiation) can be provided by a single niche, or whether different types of niches coexist. The main function of a self-renewing niche (FIG. 2b) would be to guarantee that (by environmental and/or divisional asymmetry) one of the two daughters of a dividing stem cell maintains the stem-cell fate while the other produces differentiating progenitors[53]. Such a self-renewing stem-cell niche would be more complex than a quiescent-storage niche but would be the essential unit that maintains normal tissue homeostasis. In this type of niche, one can propose that quiescent stem cells would be anchored in the centre of the niche, whereas self-renewing stem cells would be located close to the border separating the niche from the non-niche microenvironment, which could provide signals that would induce differentiation and/or cell division (FIG. 2b).

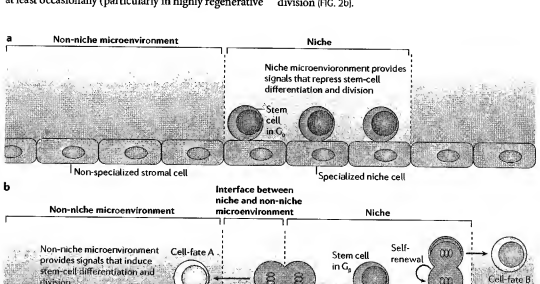

Figure 2 | **Different types of niche. a** | Quiescent-storage niche. Resting (G_0) stem cells are stored in 'quiescent' niches. Specialized niche cells generate a differentiation- and/or division-repressive environment. Under conditions of stress these might be mobilized to generate mature cells as required, and might then return to empty niches for storage or self-renewal. **b** | Self-renewing niche. Quiescent stem cells would be anchored in the centre of the niche, whereas self-renewing stem cells would be located close to the border separating the niche from the non-niche microenvironment (NNM). At this interface, niche signals (differentiation and/or division repression) and NNM signals (differentiation and/or division induction) intermingle to form a signalling centre. The appearance of a stem cell at the niche edge would expose it to proliferative and anti-adhesive signals emanating from the NNM. At the onset of cell division, one daughter cell would transit the interface towards the NNM to initiate differentiation, while the other would remain in the niche as a self-renewing stem cell, thereby achieving asymmetric division by environmental asymmetry[2,3]. Alternatively, signals from the periphery might induce asymmetric division of a formerly quiescent stem cell by polarization of determinants (right)[10]. Attachment of stem cells to niche cells (and possibly signals exchanged between them) would maintain stem-cell fate, while the budding daughter cell would initiate differentiation. Both mechanisms of asymmetrical division might occur in parallel, therefore allowing initiation of differentiation of stem cells to distinct cell fates (A and B).

Myeloablative agents
Used to completely or partially eliminate the haematopoietic system. These agents include the use of whole body irradiation or cytotoxic drugs such as 5-fluorouracil

Table 1 | **Mouse models affecting bone development and haematopoiesis**

Model	Further details of model	*In vivo* effects	Refs
Decreased number of HSCs			
Sl/Sl^d (steel-Dickie) mice	Spontaneous mutant mouse strain lacking membrane-bound SCF but not soluble SCF	Decreased osteoblast development, compromised haematopoiesis and HSC self-renewal	13,14,117
CBFα1-deficient mice	Knockout mice	No osteoblasts, no bone or bone-marrow development and no adult haematopoiesis	56,139,140
Col1α1–thymidine kinase transgenic mice	Osteoblast-specific expression of thymidine kinase allows conditional ablation of osteoblasts following administration of ganciclovir (Cytovene; Roche)	Reversible depletion of osteoblasts and haematopoietic progenitors or HSCs	69,70
Increased number of HSCs			
Intravenous administration of PTH	N/A	Increased number of osteoblasts and HSCs but not other haematopoietic cell lineages	67
Conditional BMPR1A-deficient mice	Inducible deletion of *Bmpr1a* in bone-marrow stroma	Increased number of osteoblasts and HSCs	39
Col1α1–constitutively-active-PPR transgenic mice	Osteoblast-specific expression of constitutively active PPR	Increased number of osteoblasts and HSCs	67

BMPR1A, bone morphogenetic protein receptor 1A; CBFα1, core binding factor α1; Col1α1, type 1 collagen α1; HSC, haematopoietic stem cell; N/A, not applicable; PRR, PTH/PTH-related protein receptor; PTH, parathyroid hormone; SCF, stem-cell factor.

The bone-marrow HSC niche

The link between bone-marrow formation (haematopoiesis) and bone development (osteogenesis) was first recognized in the 1970s in studies showing that first bone and then vascularized bone marrow developed after subcutaneous transfer of total, unmanipulated bone marrow[24,25]. The term 'niche' for the specific HSC bone-marrow microenvironment was first coined by Schofield, who proposed that HSCs are in intimate contact with bone, and that cell–cell contact was responsible for the apparently unlimited proliferative capacity and inhibition of maturation of HSCs[11].

More recently, several mutant mice in which haematopoiesis is defective as a consequence of primary defects in bone development or remodelling, have implicated osteoblasts and/or osteoclasts in the formation and function of the bone-marrow HSC environment or niche (TABLE 1). For example, mice lacking core binding factor α1 (CBFα1; also known as RUNX2), which is one of the earliest osteoblast-specific transcription factors, have defective bone-marrow haematopoiesis and extensive extramedullary haematopoiesis, owing to defects in osteoblast differentiation and the consequent failure to form bone[56,57] (see Supplementary information S1 (table)). However, whether the haematopoietic deficiency is a secondary effect caused by the absence of a suitable bone-marrow cavity or whether the deficiency in CBFα1 directly affects haematopoiesis remains unclear. In this respect, several other mouse mutants with defects in bone development and/or remodelling have been described[58] but potential effects on haematopoiesis have not been documented.

The physical location of HSCs close to the bone surface was first shown in 1975 (REF. 59). Morphological evidence for the presence of HSC niches in close association with the endosteum was provided more recently when HSC or haematopoietic progenitor activity and/or phenotype (TABLE 2) were shown to localize close to the endosteal lining of bone-marrow cavities in trabecular regions of long bones, whereas more differentiated haematopoietic progenitors were found mainly in the central bone-marrow region[9,39,52,59–63]. For example, 89% of CD45$^+$Lin$^-$ LRCs were shown to be attached to the endosteal surface, and only 11% of these cells were in the centre of the bone marrow[39]. However, in another study, although 57% of a bone-marrow population highly enriched in LTR activity and defined as CD150$^+$CD48$^-$CD41$^-$Lin$^-$ (denoted CD150$^+$ HSCs) were located in trabecular bone, only 14% were found at the endosteum and the rest were found at bone-marrow sinusoids (as discussed later)[9].

The discrepancy between these two studies[9,39] might be a reflection of the different criteria used to identify HSC subsets *in situ*, which could mean that the populations contain different proportions of functional, or quiescent versus self-renewing, HSCs (TABLE 2). Nevertheless, HSCs are likely to be located in close proximity to bone surfaces, supporting the concept of an endosteal niche.

The endosteal bone-marrow HSC niche. The first direct evidence for cells involved in bone formation having stem-cell-supporting activity was provided by studies in which both mouse and human osteoblast cell lines were shown to secrete a large number of cytokines that promote the proliferation of haematopoietic cells in culture[64]. Furthermore, long-term bone-marrow cultures contain osteoblasts, and many, but not all, stromal cell lines that have been shown to maintain HSCs *in vitro* also show bone-formation activity[64–66].

A direct role for the involvement of osteoblasts in HSC regulation and/or maintenance *in vivo* has recently been obtained from two studies in which osteoblast numbers were experimentally increased or decreased. In the first study[67], osteoblast-specific expression of a constitutively active form of parathyroid hormone (PTH) or the PTH/PTH-related protein receptor (PPR), which is an important regulator of calcium homeostasis, and therefore bone formation and resorption, was achieved using the type 1 collagen α1 (*Col1a1*) promoter. This

Osteoblasts
Mesenchymal cells that produce bone matrix that forms bone after mineralization

Osteoclasts
Large, multi-nucleated cells derived from macrophages that resorb bone. The activity of osteoblasts and osteoclasts form an equilibrium that maintains bone during homeostasis and remodelling

Endosteum
The cellular lining separating bone from bone marrow. It comprises different cell types including osteoblasts, osteoclasts and stromal fibroblasts

Bone-marrow sinusoids
Low-pressure vascular channels surrounded by a single layer of fenestrated endothelium

Table 2 | **Localization of HSCs or haematopoietic progenitor cells in the bone marrow**

Cell population	Source*	Assay	Result	Refs
Femoral bone marrow	Endogenous	CFUs	• 3-fold increase in CFU activity in endosteal bone marrow compared with central bone marrow	59
Endosteal bone-marrow cells identified as Lin⁻ Sudan-Black staining, and with lymphoid morphology	Endogenous	Scanning electron microscopy and histology of opened rat bone. CFUs of endosteal bone marrow	• Lin⁻ Sudan-Black-staining cells morphologically resembling lymphocytes located close to endosteum • High frequency of CFUs from endosteal bone marrow	60
Lin⁻ bone marrow	Transplanted	*In situ* localization of CFSE-labelled cells transferred into sub-lethally irradiated hosts	• 15h post-transplantation CFSE-labelled cells attached to BMPR1A⁺OPN⁺N-cad⁺ osteoblasts at endosteum	52
Lin⁻ Rho^{low} WGA$^{low/-}$ bone marrow	Transplanted	*In situ* localization of CFSE-labelled cells 10–15h post transfer into non-ablated recipients	• >60% CFSE-labelled cells at endosteum post-transplantation	53
LSK HSCs mixed with Lin⁻SCA1⁺ and Lin⁻KIT⁺ (HSPCs)	Transplanted	Intravital bone-marrow imaging (calvarial bone) of transferred DiR-labelled cells	• Attachment of HSPCs to CXCL12⁺ vascular microdomains in the perivascular space 2h after transfer • No quantitation	62
Lin⁻CD45⁻ LRCs (BrdU⁺). At least 50% are also SCA1⁺KIT⁻	Endogenous	*In situ* localization by immuno-histochemistry	• 6-fold increase of BrdU⁺Lin⁻CD45⁻ LRCs at endosteum compared with bone marrow centre • LRCs attached to N-cad⁺ osteoblasts	39
Lin⁻SCA1⁺CD41⁻CD45⁻CD48⁻ CD150⁺ bone marrow or mobilized spleen	Endogenous	*In situ* localization by immuno-histochemistry	• 57% in trabecular bone, 14% at endosteum • 60% associated with MECA-32⁺ sinusoidal epithelium in bone marrow • 100% in contact with or close to MECA-32⁺ sinusoidal epithelium in mobilized spleen	9

*In situ detection of endogenous or transplanted HSCs or haematopoietic progenitor cells. BMPR1A, bone morphogenetic protein receptor 1A; BrdU, bromodeoxyuridine; CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester; CFUs, colony-forming units in the spleen; CXCL12, CXC-chemokine ligand 12; DiR, diallylcarbocyanine; HSPCs, haematopoietic stem and progenitor populations; Lin⁻, lineage negative; LRCs, label-retaining cells; LSK, Lin⁻SCA1⁺KIT⁺ cells; MECA-32, a pan-endothelial marker; N-cad, N-cadherin; OPN, osteopontin; Rho, rhodamine 123; SCA1, stem-cell antigen 1; WGA, wheat germ agglutinin.

resulted in a simultaneous increase in the number of both osteoblasts and HSCs in the bone marrow. Moreover, the maintenance of HSCs *in vitro* was more efficient when supported by stromal cells that were isolated from these transgenic mice, presumably because of an increase in the proportion of osteoblasts in the stromal-cell population compared to stromal cells from wild-type mice[67].

In a second study[39], mice lacking bone morphogenetic protein (BMP)[68] receptor 1A (BMPR1A, which is normally expressed on osteoblasts lining the endosteum) in the bone-marrow stroma showed a simultaneous increase in the number of both osteoblasts and repopulating HSCs, although the number of more differentiated cells remained unchanged[39]. Moreover, Lin⁻ LRCs and osteoblasts were shown to be in direct contact through homotypic N-cadherin interactions (TABLE 2). Therefore, specialized spindle-shaped N-cadherin-expressing osteoblasts (SNOs) located in the endosteum were postulated to be essential components of the HSC bone-marrow niche (FIG. 3). Both studies[39,67] show that an increase in the number of osteoblasts directly correlates with the number of functional LTR HSCs, indicating that osteoblasts (or a subset of these cells) are an essential part of the niche and are limiting for niche size and/or activity. This concept is supported by experiments in which osteoblasts were conditionally ablated by targeting the expression of thymidine kinase (which induces cell death in response to ganciclovir (Cytovene, Roche), to the osteoblast lineage[69,70]. In these mice, progressive bone loss is accompanied by a decrease in bone-marrow cellularity, including a decrease in the number of LSK HSCs. Importantly, in response to the loss of osteoblasts, progenitor cells (and presumably HSCs) are now found in the liver, spleen and peripheral blood. This type of extramedullary haematopoiesis is a typical response to bone-marrow stress. Interestingly, osteoblast depletion due to thymidine-kinase activity is reversible following the removal of ganciclovir and is accompanied by a corresponding re-emergence of bone-marrow haematopoiesis. By contrast, genetic ablation of osteoblasts using the osteocalcin promoter (which is active at a later stage of osteoblastogenesis than the *Col1a1* promoter) to drive thymidine-kinase expression has no effect on haematopoiesis[71], indicating that niches comprise immature osteoblasts. Although N-cadherin-expressing PPR⁺BMPR1A⁺ osteoblasts seem to be necessary and rate-limiting for niche function, it is probable that other cell types, such as osteoclasts, stromal fibroblasts and endothelial cells, also contribute to niche formation, activity or architecture.

The vascular bone-marrow HSC niche. The presence of a second specialized HSC microenvironment in the bone marrow has recently been postulated, as a large proportion of CD150⁺ HSCs were observed to be attached to the fenestrated endothelium of bone-marrow sinusoids[9] (TABLE 2). A close interaction between HSCs and endothelial cells is not unexpected because both lineages arise from a common embryonic precursor, the haemangioblast[72]. Moreover, cell lines or purified primary endothelial cells that are derived from the yolk sac or the aorta–gonad–mesonephros promote the maintenance, or even clonal expansion,

Bone morphogenetic protein
Induces the formation of bone and cartilage, and is a member of the transforming growth factor β (TGFβ) superfamily

Stromal fibroblasts
Part of the endosteal lining separating bone and bone marrow

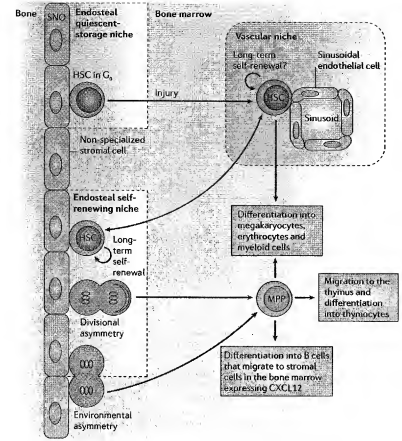

Figure 3 | **Model of bone-marrow HSC niches.** Endosteal bone surfaces are lined with stromal cells. Spindle-shaped N-cadherin-expressing osteoblasts (SNOs) serve as niche cells to maintain quiescence and prevent differentiation of attached haematopoietic stem cells (HSCs). The quiescent endosteal niche would maintain dormant HSCs long-term. In response to injury, quiescent HSCs might be activated and recruited to the vascular niche. The self-renewing niche would contain quiescent HSCs intermingled with dividing HSCs. Self-renewing HSCs produce multipotential progenitors (MPPs) either by divisional or environmental asymmetry. More HSCs can be generated by symmetrical divisions which might provide the vascular niche with new HSCs. Whether HSCs long-term self-renew in the vascular niche remains to be determined, and it is probable that influx of HSCs from endosteal niches is necessary to ensure prolonged haematopoietic-cell production at the vascular niche. HSCs in the vascular niche promote differentiation and expansion along megakaryocytic and other myeloid-cell lineages, particularly in response to injury. MPPs can give rise to all haematopoietic lineages, including B-cell precursors attached to randomly distributed CXC-chemokine ligand 12 (CXCL12)-expressing stromal cells that constitute a B-cell niche[82]. Unidentified T-cell precursors migrate to the thymus where they enter a microenvironment, promoting T-cell maturation.

A vascular bone-marrow HSC niche has previously been predicted to form during HSC mobilization after myeloablation. Quiescent HSCs detach from the endosteal niche and migrate towards the centre of the bone marrow to the vascular zone from where they re-establish haematopoiesis[76,77,79]. The recent finding that $CD150^+$ HSCs are attached to the sinusoidal endothelium now raises the possibility that a vascular bone-marrow HSC niche might also exist during homeostasis[9]. Why have two apparently distinct HSC niches in the bone marrow? Putative HSCs that have been identified by LRC assays are almost exclusively located in the endosteal niche[39], indicating that this niche might contain the most dormant HSCs and therefore serve as a quiescent-storage niche, or a self-renewing niche comprising both quiescent and self-renewing HSCs. In contrast to label-retaining HSCs that have not divided for many weeks, the $CD150^+$ HSC population comprises both long-term quiescent and self-renewing HSCs, because 3.8% of the cells are proliferating at any given time[9]. Because many of the proliferating cells are in contact with BMECs, it is probable that the vascular bone-marrow HSC niche contains self-renewing, rather than long-term dormant, HSCs. The location of $CD150^+$ HSCs — in close proximity to sinusoids — would enable them to constantly monitor the concentration of blood-borne factors that reflect the status of the haematopoietic system. Under haematological stress, a rapid and robust response could be mounted, and if necessary more HSCs could be recruited from endosteal niches (FIG. 3).

BMECs are known to support the survival, proliferation and differentiation of myeloid and megakaryocyte progenitors[76,77,80], whereas primary bone-marrow stromal cells release factors that inhibit megakaryocyte maturation[81]. These data indicate that megakaryocyte lineage development (and possibly the development of other myeloid-cell types) might be predominantly initiated at the vascular niche[76] (FIG. 3). It is probable that the pool of HSCs located in the vascular and self-renewing endosteal niches are freely exchanged to maintain homeostasis in a constantly changing haematopoietic environment. In addition, HSCs that are located in the self-renewing endosteal niche produce multipotential progenitors (MPPs) by divisional and/or environmental asymmetry (FIG. 3). These cells give rise to myeloid-cell lineages as well as lymphocyte precursors. B-cell progenitors are uniformly distributed throughout the bone marrow attached to CXCL12-expressing fibroblasts (in the B-cell niche)[82,83]. Because deletion of osteoblasts results in extramedullary haematopoiesis[70], the vascular bone-marrow HSC niche alone might not be sufficient to maintain long-term haematopoiesis. This indicates that in the bone marrow the vascular niche might be a secondary niche, requiring an influx of HSCs from the primary endosteal niches (FIG. 5). Collectively, the vascular and endosteal niches strongly cooperate to control HSC quiescence and self-renewing activity (and therefore HSC number), as well as the production of early progenitors to maintain homeostasis or re-establish it after injury.

Mobilization
The efflux of haematopoietic stem cells from the bone marrow into the vasculature in response to bone-marrow stress or injury, or after treatment with cytokines such as granulocyte colony-stimulating factor (G-CSF).

of adult LSK HSCs *in vitro*[73,74]. By contrast, vascular endothelial cells that are isolated from various adult non-haematopoietic organs have little or no ability to maintain HSCs *in vitro*[75]. Therefore, bone-marrow sinusoidal endothelial cells (BMECs) are functionally and phenotypically distinct from microvasculature-endothelial cells of other organs[76]. Indeed BMECs constitutively express cytokines such as CXC-chemokine ligand 12 (CXCL12) and adhesion molecules such as endothelial-cell (E)-selectin and vascular cell-adhesion molecule 1 (VCAM1) that are important for HSC mobilization, homing and engraftment[62,77,78] (FIG. 4).

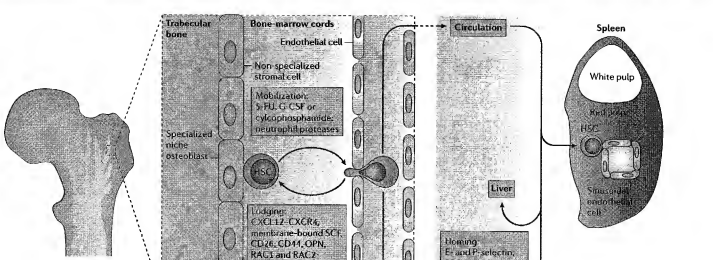

Figure 4 | **Mobilization, homing and lodging.** Schematic diagram showing some of the factors implicated in each process. Haematopoietic stem cells (HSCs) bound to the bone-marrow niche are mobilized in response to granulocyte colony-stimulating factor (G-CSF) or cyclophosphamide, or after peripheral myeloablation following treatment with 5-fluorouracil (5-FU). After extravasation from the bone-marrow cords into the microvasculature, HSCs enter the circulation and are distributed to peripheral tissues such as the spleen or liver. HSCs locate close to endothelial cells in the splenic red pulp. They home to the bone-marrow cords through the circulation, a process that is controlled by a number of adhesion molecules such as very late antigen 4 (VLA4), VLA5, lymphocyte function-associated antigen 1 (LFA1) or selectins. After entering the bone marrow, HSCs specifically lodge in the niche, a process requiring membrane-bound stem-cell factor (SCF), CXC-chemokine ligand 12 (CXCL12), osteopontin (OPN), hyaluronic acid, and their corresponding receptors. CXCR4, CXC-chemokine receptor 4; E-selectin, endothelial-cell selectin; P-selectin, platelet selectin; PSGL1, P-selectin glycoprotein ligand 1.

Entering and exiting the HSC niche

Although the vast majority of HSCs in the adult mouse are located in the bone marrow, HSCs show remarkable motility. In response to specific signals they can exit and re-enter the endosteal bone-marrow HSC niche, processes known as mobilization and homing, respectively (FIG. 4). These opposing biological processes are controlled by overlapping but distinct molecular mechanisms[84–86]. Massive mobilization of HSCs occurs in response to treatment with cyclophosphamide and granulocyte colony-stimulating factor (G-CSF), or bone-marrow injury. This is mediated by the release of neutrophil proteases, which lead to the degradation of niche-retention signals and adhesive connections (such as those provided by membrane-bound SCF, VCAM1 and CXCL12)[87] (FIG. 4). During extramedullary haematopoiesis (which can occur in the liver and spleen), extramedullary long-term self-renewal of HSCs might occur, so HSC niches should also be present in the spleen. Support for this idea comes from immunohistochemical analysis showing that two out of three mobilized CD150$^+$ HSCs in the spleen are in contact with sinusoidal endothelial cells[9]. Whether mobilized HSCs are retained in these locations in situations of sustained extramedullary haematopoiesis, and whether these niches are functionally equivalent to those present in the bone marrow, remains to be shown.

The release of HSCs not only occurs during mobilization but is also observed during homeostasis, when a small number of HSCs are constantly released into the circulation[88]. Although their precise physiological role remains unclear, they might provide a rapidly accessible source of HSCs to repopulate areas of injured bone marrow[89]. Alternatively, circulating HSCs might be a secondary consequence of permanent bone remodelling that causes constant destruction and formation of HSC niches, therefore requiring frequent re-localization of HSCs.

Transplanted HSCs have the capacity to home back to and lodge in bone-marrow niches. Homing can be defined as recruitment of circulating HSCs to the bone-marrow microvasculature and subsequent transendothelial migration into the extravascular haematopoietic cords of the bone marrow[13] (FIG. 4). Several cell-surface adhesion molecules, including selectins and integrins, are crucial for homing of HSCs to the bone-marrow HSC niche[84,85]. For example, β_1-integrin-deficient HSCs fail to migrate to the bone marrow after transfer[19]. Although homing is thought to be an unselective process that occurs at a similar frequency for most haematopoietic cell types, transendothelial migration into the extravascular haematopoietic cords of the bone marrow and subsequent lodging in endosteal bone-marrow HSC niches is a specific property of HSCs[15,90,91]. Subsequent engraftment is accompanied by the generation of a large number of haematopoietic progenitors and differentiated cells.

One crucial factor involved in migration, retention and mobilization of HSCs during homeostasis and after injury is CXCL12 (FIG. 4), which is expressed by several types of bone-marrow stromal cell, including osteoblasts

Homing
The specific movement or migration of haematopoietic stem cells through the vasculature to the bone marrow.

Engraftment
The production of more haematopoietic stem cells by symmetrical divisions and production of a large number of progenitors and differentiated cell types.

LRC assay
(Label retaining cell assay). Identifies long-lived quiescent cells such as adult stem cells. They can be visualized *in situ* by pulse labelling of their DNA with BrdU (or ^{3}H-thymidine or a histone H2B–EGFP transgene) followed by a chase period of a month or more. Detection of BrdU$^+$ cells requires fixation, precluding subsequent functional analysis.

and vascular endothelial cells[90,93]. Similar to SCF, CXCL12 expression and secretion is induced in response to haematopoietic-cell loss due to irradiation, chemotherapy or hypoxia, and purified HSCs migrate specifically towards CXCL12 but not towards any other single chemokine[94]. The biological effects of CXCL12 are mediated by its capacity to induce motility, chemotaxis and adhesion, as well as to induce secretion of matrix metalloproteinases (MMPs) and angiogenic factors (such as vascular endothelial growth factor (VEGF)) by cells expressing its receptor, CXC-chemokine receptor 4 (CXCR4). Mice lacking either CXCL12 or CXCR4 show similar embryonic lethal defects, including impaired myeloid- and B-cell haematopoiesis[87,95,96] (see Supplementary information S1 (table)). Importantly, CXCL12 is not essential for HSC generation or expansion in the fetal liver but is crucial for the colonization of bone marrow during late fetal development. Collectively, the genetic and functional data indicate that the CXCL12–CXCR4 pathway is crucial for retention and maintenance of adult HSCs.

The cytoskeleton also cooperates with cell-surface adhesion molecules to regulate migration and adhesion, and is essential for homing and mobilization. For example, Lin$^-$KIT$^-$ cells lacking the RHO family GTPase RAC1 not only fail to engraft[97], but also have reduced homing efficiency to the bone marrow and endosteum. Moreover, the deletion of both RAC1 and RAC2 causes massive defects in HSC or haematopoietic-progenitor-cell proliferation, survival, adhesion to very late antigen 4 (VLA4) and/or VLA5, and migration towards CXCL12 *in vitro*. Deletion of both RAC1 and RAC2 in engrafted HSCs *in vivo* leads to a massive mobilization of HSC or haematopoietic progenitor cells to the peripheral blood. Together, these data indicate that RAC1 and RAC2 have essential roles in homing, lodging and retention of HSCs in the endosteal bone-marrow HSC niche[86,97] (see Supplementary information S1 (table)). In summary, a complex combination of migration, adhesion, proteolysis and signalling occurs at the interface between HSCs and the endosteal bone-marrow niche (FIGS 4,5), and signals originating from the periphery can influence HSC homing, retention and mobilization, therefore determining whether a niche is silent or whether HSCs exit the niche in response to stress.

Molecular crosstalk in the endosteal niche

Although little is known about the signals that are exchanged between HSCs and osteoblasts *in situ*, several receptors, membrane-anchored proteins and secreted factors are expressed by both cell types[66]. Comparative gene-expression profiling has recently been performed on HSC-supporting and non-supporting stromal cell lines, identifying a number of new molecules that might regulate endosteal bone-marrow HSC-niche activity. These include various interleukins, oncostatin-M, ciliary neurotrophic factor and the membrane protein mKirre[98]. Here, however, we will focus on the role of molecules for which genetic or functional evidence has been shown *in vivo* for the regulation of HSC function and/or niche activity (FIG. 5) (see Supplementary information S1 (table)).

Angiogenic factors
These factors (which include angiopoietin 1) promote the development of blood vessels, and are particularly important in embryonic and fetal development.

Notch signalling. Signalling through Notch receptors is involved in many cell-fate decisions and is thought to have a role in the maintenance of stem cells in a variety of tissues[99,100]. Moreover, several Notch receptors and Notch-receptor ligands are expressed in the bone marrow[101], leading to the suggestion that Notch signalling has a role in HSC self-renewal and/or clonal expansion. Support for this hypothesis has been provided by *in vitro* culture of purified HSCs on various stromal cell lines[102,103]. In addition, overexpression of Notch1 in recombination-activating gene 1 (RAG1)-deficient Lin$^-$SCA1$^+$ progenitors resulted in an increase in the number of HSCs or haematopoietic progenitor cells *in vitro* and *in vivo*[104]. Moreover, as expression of the Notch ligand Jagged-1 is upregulated on osteoblasts that are exposed to PTH, the concomitant increase in HSCs has been postulated to be caused by increased Notch signalling[67]. However, in contrast to studies leading to over-activation of Notch signalling, loss-of-function studies have failed to show any requirement for Notch signalling in HSCs. Conditional knockout mice for Jagged-1, Notch-1 and Notch-2, or CSL (the common mediator of all signalling through Notch receptors) have all been shown to be dispensable for HSC and niche function *in vivo*[105–108]. Together, these data indicate that signalling events occurring between HSCs and osteoblasts are more complex than has been previously assumed and involve factors other than Notch signals.

Osteopontin. One mechanism by which osteoblasts might regulate the number of HSCs in the bone marrow is through secretion of osteopontin (OPN), an acidic glycoprotein, into the bone matrix[109]. OPN-deficient mice have a two-fold increase in HSCs and, because the same effect was observed by transplanting wild-type HSCs into lethally irradiated OPN mutant recipients, OPN production by osteoblasts has a negative effect on HSC number[91,110]. Because cultured Lin$^-$SCA1$^+$ bone-marrow cells are induced to undergo apoptosis when exposed to soluble OPN, the increase in the number of HSCs in OPN-deficient mice has been postulated to be a result of decreased apoptosis[110]. In addition, OPN has been postulated to act as a negative regulator of HSCs by actively maintaining their quiescence[91].

Membrane-bound SCF. The steel (*Sl*) locus encodes both membrane-bound SCF and secreted SCF. The latter is produced by alternative splicing followed by proteolytic cleavage of membrane-bound SCF[111]. SCF binds and activates KIT, which is expressed at high levels by all LTR HSCs as well as other stem cells. Mutations at either of these loci affect migration and differentiation of primordial germ cells, neural-crest-derived melanoblasts, and haematopoietic cells[112] (see Supplementary information S1 (table)). Analysis of the different SCF and KIT mutant mice showed that although not essential for the generation and initial clonal expansion of HSCs in the embryo and fetal liver, they are crucial for long-term maintenance and self-renewal of adult HSCs, raising the possibility that the SCF–KIT pathway mediates endosteal bone-marrow HSC niche activity (FIG. 5).

Importantly, membrane-bound SCF is expressed by osteoblasts and has a higher and more sustained capacity to activate KIT on the cell surface of HSCs than secreted SCF[112,113]. In addition, membrane-bound SCF is a potent stimulator of adhesion of HSCs or haematopoietic progenitor cells to stromal cells[114] because it can activate VLA4 and VLA5, indicating that membrane-bound SCF can affect the adhesive properties of the endosteal niche by modifying the functional state of specific integrins[115]. Transplantation of normal bone marrow into *Sl/Sl*^{d} mice results in impaired lodging and engraftment of the transplanted HSCs[12,116]. In addition, the bone marrow of young *Sl/Sl*^{d} mice has normal LTR activity when transplanted into lethally irradiated recipients, whereas bone marrow from old *Sl/Sl*^{d} mice has greatly reduced LTR activity, indicating a progressive loss of HSC activity over time, potentially due to ceasing niche activity[13,14]. Collectively, these data indicate that membrane-bound SCF is an essential component of the endosteal bone-marrow HSC niche that maintains long-term HSC activity in adult bone marrow. However, membrane-bound SCF is also required for osteoblast proliferation and activity *in vivo*, as shown by the development of osteopaenia in *Sl/Sl*^{d} mice[117] (TABLE 1). Therefore, further research is required to clarify whether the effect of membrane-bound SCF is direct (due to its capacity to provide sustained activation of KIT expressed by HSCs), or whether it is indirect (owing to its essential role in the maintenance of niche osteoblasts).

N-cadherin: a central HSC anchor? N-cadherin is expressed by both SNOs and a subset of LSK HSCs[38,52]. In addition, N-cadherin expression by HSCs localizes asymmetrically to the side of their attachment to SNOs[39].

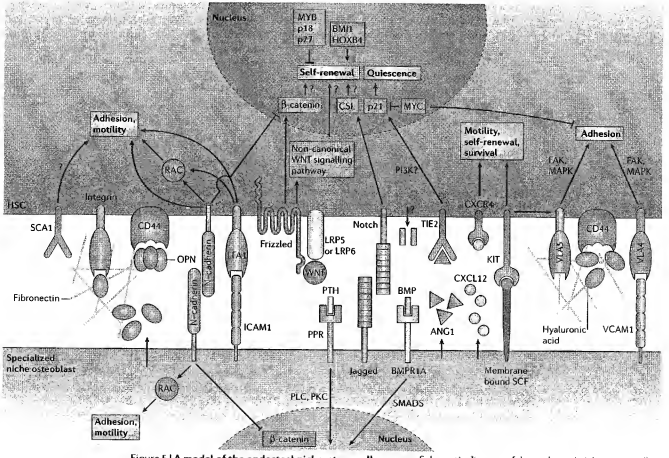

Figure 5 | **A model of the endosteal niche–stem-cell synapse.** Schematic diagram of the endosteal niche–stem-cell synapse showing putative ligand–receptor interactions and adhesion molecules, as well as some of the intracellular pathways that are activated following signalling. ANG1, angiopoietin-1; BMI1, polycomb repressor; BMP, bone morphogenetic protein; BMPR1A, BMP receptor 1A; CSL, CBF1 suppressor of Hairless and LAG1; CXCL12, CXC-chemokine ligand 12; CXCR4, CXC-chemokine receptor 4; FAK, focal adhesion kinase; HOXB4, homeobox B4; HSC, haematopoietic stem cell; ICAM1, intercellular adhesion molecule 1; LFA1, lymphocyte function-associated antigen 1; LRP, low-density-lipoprotein-receptor-related protein; MAPK, mitogen-activated protein kinase; OPN, osteopontin; PI3K, phosphatidylinositol-3 kinase; PLC, phospholipase C; PKC, protein kinase C; PPR, PTH/PTH-related protein receptor; PTH, parathyroid hormone; SCF, stem-cell factor; SMADS, mothers against decapentaplegic-related homologue; SNO, spindle-shaped N-cadherin-expressing osteoblast; TIE2, tyrosine kinase receptor 2; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4; '?' denotes molecules and/or interactions for which only indirect or contradictory evidence is available.

Therefore, homotypic N-cadherin interactions have been postulated to be an important component of the anchor that links HSCs to SNOs in the endosteal niche.

In support of this hypothesis, ectopic expression of N-cadherin by OP9 stromal cells substantially increases their ability to maintain mouse HSCs *in vitro*[95]. However, genetic evidence of an essential role for N-cadherin in HSC–osteoblast adhesion and/or signalling is still lacking, as N-cadherin-mutant embryos fail to develop past mid-gestation[114]. Moreover, whether functional HSCs are enriched in N-cadherin-expressing LSK HSCs, compared to those not expressing this adhesion receptor, has not been shown. Nevertheless, indirect support for the importance of N-cadherin has been obtained from studies showing that MYC and tyrosine kinase receptor 2 (TIE2) control N-cadherin expression by HSCs in an antagonistic manner. The effects of MYC and TIE2 on HSCs and on N-cadherin expression correlate with a key function for N-cadherin in the retention of HSCs in the endosteal niche[83,85,92,93].

The cell-adhesion signalling network. Genetic evidence for the requirement of TIE2 in HSC–niche interactions has been obtained from chimeric mice comprised of wild-type and *TIE1*$^{-/-}$*TIE2*$^{-/-}$ morulae[119]. Although TIE1 and TIE2 are not required for the development and differentiation of fetal HSCs, HSCs lacking both TIE1 and TIE2 fail to be maintained in the adult microenvironment. In adult bone marrow, TIE2 (which is expressed specifically by LT-HSCs) is activated by angiopoietin-1 (ANG1), which is secreted by osteoblasts, leading to upregulation of N-cadherin expression by HSCs, providing the first example of a secreted factor promoting HSC–osteoblast adhesion. Interestingly, the ANG1–TIE2 signalling pathway prevents HSC division and maintains HSC quiescence, both *in vitro* and *in vivo*[36,51,65,120]. Collectively, these data strongly support the hypothesis that N-cadherin-expressing ANG1^{+} osteoblasts form a niche that maintains quiescence and prevents self-renewal or differentiation through TIE2 signalling (FIG. 5).

TIE2-mediated quiescence is potentially caused by positively regulating the cyclin-dependent-kinase inhibitor p21 (also known as CIP1 and WAF1). HSCs express high levels of p21, and mice lacking p21 show increased HSC proliferation at the expense of long-term self-renewal, indicating that p21 is essential for maintenance of quiescence in HSCs[120,121] (see Supplementary information S1 (table)). In contrast to TIE2, transcription of the gene encoding p21 is negatively regulated by MYC, which is expressed at low levels by HSCs but increases during initiation of HSC differentiation in a converse expression pattern to that of p21 (REFS 52,121,122). Interestingly, MYC-deficient LSKFLT3^{-} HSCs overexpress N-cadherin and integrins such as lymphocyte function-associated antigen-1 (LFA1) and VLA5, and contact SNOs[52]. Although mutant LSKFLT3^{-} HSCs self-renew normally, they have a severe niche-dependent differentiation defect and accumulate *in situ*. Conversely, enforced MYC activity in HSCs represses the expression of N-cadherin, as well as the expression of several integrins, by LSK HSCs. Most importantly, MYC overexpressing HSCs are lost over time because of differentiation, presumably owing to their failure to be retained in the niche[52]. These data indicate that the balance between self-renewal and differentiation might be controlled by MYC-dependent retention or exit of HSCs from the niche[52,53].

N-cadherin and WNT signalling. Intriguingly, it has recently been shown that the transmembrane metalloproteinase ADAM10 (a disintegrin and metalloproteinase-10) is able to cleave N-cadherin that is expressed at the cell surface of fibroblasts and neuronal cells. This leads to the redistribution of β-catenin (which is associated with the intracellular portion of N-cadherin) from the cell surface to the cytoplasmic β-catenin pool, thereby decreasing the signalling threshold required for the expression of target genes of the canonical WNT signalling pathway (which is mediated through β-catenin signal transduction cascades), such as the genes encoding cyclin D1 and MYC[123]. A similar re-distribution of β-catenin has also been reported after E-cadherin cleavage[124], indicating that high levels of expression of cadherins, as observed for HSCs, might decrease cytoplasmic β-catenin levels and therefore negatively regulate expression of β-catenin target genes.

This contrasts with studies in which activation of the WNT signalling pathway in cultured HSCs promotes symmetrical self-renewal in the absence of differentiation[125,126]. However, the importance of canonical WNT signalling during haematopoiesis has recently been questioned because β-catenin is dispensable for HSC function[127]. Although it is probable that the WNT signalling pathway has an important role in HSC or haematopoietic progenitor cell function[100], the question remains whether WNT promotes self-renewal of LT-HSCs *in vivo*, or whether it is only important for the expansion and differentiation of non-HSC haematopoietic progenitor cells. The latter is in agreement with the expression pattern of the β-catenin target gene *Myc*, which is induced in LSKFLT3^{+} progenitor cells leading to downregulation of N-cadherin and integrin expression[52]. In this context, it is intriguing that the N-cadherin, TIE2, MYC, p21 and β-catenin pathways are apparently interconnected, leading to the suggestion that they might cooperatively control quiescence, self-renewal and initiation of HSC differentiation through interaction with the niche[53] (FIG. 5 and Supplementary information S1 (table)).

The stem-cell–niche synapse

The picture emerging from accumulating genetic and functional data indicates that molecular crosstalk between HSCs and niche cells (particularly osteoblasts) involves a large number of molecules (cadherins, integrins, chemokines, cytokines, signalling molecules and receptors) that mediate at least two types of interaction (FIG. 5). First, adhesive cell–extracellular-matrix (ECM) interactions such as CD44 binding to OPN or hyaluronic acid, and cell–cell interactions, such as those mediated by heterotypic VLA4–VCAM1 interactions and homotypic N-cadherin interactions. The main function of these interactions would be to maintain HSCs in close

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proximity to cells in the endosteal bone-marrow niche. In addition, most adhesion receptors are also linked to intracellular signalling cascades and actively participate in the signalling network controlling HSC maintenance (FIG. 5). Second, ligand–receptor interactions, through which intracellular signalling pathways are activated after ligand binding to receptors that are expressed by HSCs or SNOs (FIG. 5).

Most secreted signalling molecules are bound to the cell surface or ECM, and consequently do not diffuse far. Therefore, the tight adhesion and juxtaposition of HSCs to niche osteoblasts is essential for the formation of an intercellular space in which efficient ligand–receptor interaction can occur. Some osteoblast-derived signals might be crucial to maintain HSCs in an undifferentiated state and these include the ligand–receptor pairs membrane-bound SCF–KIT, and ANG1–TIE2–MYC (see Supplementary information S1 (table)). Conversely, other ligand–receptor pairs, such as BMP–BMPR1A are important for the number and/or activity of niche osteoblasts. Therefore, in analogy to the neuronal and immunological synapses[128], we propose the term 'stem-cell–niche synapse' for this adhesion and signalling unit (FIG. 5).

Concluding remarks

During the past few years, the theoretical concept of a specific stem-cell microenvironment (that is, a stem-cell niche) that was proposed in the 1960s and 1970s, has finally received greater attention[129]. Substantial progress in localizing the bone-marrow HSC niche(s), as well as its characterization at the molecular and cellular levels, has been made. Nevertheless, important questions remain. These include, how many different types of haematopoietic niches exist in the bone marrow and the periphery, how many HSCs each niche contains, and the exact role each niche unit has during homeostasis and in response to bone-marrow stress. It also remains unclear whether HSC–niche interactions are stable or dynamic. Furthermore, although osteoblasts have been shown to be rate-limiting for HSC number, very little is known about the specific differentiation stage of these cells. Are they the same mesenchymal-stem-cell-derived osteoblasts that continue to differentiate into osteocytes, or have they branched off to generate a distinct 'niche-osteoblast'? If the latter is the case, do they differentiate in response to signals that are derived from an attaching HSC? The recently identified vascular niche[9] opens another chapter on HSC–bone-marrow-niche interactions, and the molecular events governing adhesion and signalling of BMECs with HSCs will be an area of intense future research and will move the endothelial-cell field to one of the centre stages of adult-stem-cell research.

Finally, whether long-term self-renewal occurs in sites of extramedullary haematopoiesis, such as the spleen and liver, and therefore maintains blood formation during acute and chronic bone-marrow injury, remains unclear. First attempts to address this question indicate that mobilized splenic HSCs are found close to the vasculature (peripheral vascular niche)[9]. Are these areas active niches or do they only transiently maintain HSCs? Are they always present or do they form only after injury, and what are the equivalent niche structures in the liver? At the moment there are more questions than answers, but a better understanding of the different niches will also unearth similarities between them, which should facilitate the eventual reconstruction of active niches *in vitro*.

Collectively, the impressive progress in the HSC-niche field clearly indicates that substantial clonal expansion of HSCs *in vitro* unquestionably requires more than just a cytokine cocktail, and instead requires a three-dimensional reconstruction of the niche, including the appropriate cells and ECM to allow the generation of a stable 'stem-cell–niche synapse'. This requires not only further progress from the cell–and-molecular-biology end, but is in urgent need of input from matrix- and tissue-engineering fields. Future perspectives have never been more promising, and a breakthrough in the *in vitro* expansion field will eliminate one of the main obstacles for future regenerative medicine using adult stem cells[130].

Note added in proof

A recent report shows that HSCs that are deficient for the calcium-sensing receptor show decreased homing to the endosteal niche accompanied by diminished adhesion to collagen type I. These data indicate that local calcium gradients, as are observed around areas of bone remodelling, might be involved in engraftment and/or retention of HSCs to the endosteal niche[131].

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Competing interests statement

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The *Hox11* gene is essential for cell survival during spleen development

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SUMMARY

The *HOX11* homeobox gene was identified via the translocation t(10;14) in T cell leukaemia. To determine the function of this gene in mice, null mutations were made using homologous recombination in ES cells to incorporate *lacZ* into the *hox11* transcription unit. Production of β-galactosidase from the recombinant *hox11* allele in +/– mutants allowed identification of sites of hox11 expression which included the developing spleen. Newborn *hox11* –/– mice exhibit asplenia. Spleen formation commences normally at E11.5 in *hox11* –/– mutant embryos but the spleen anlage undergoes rapid and complete resorption between E12.5 and E13.5. Dying spleen cells exhibit molecular features of apoptosis, suggesting that programmed cell death is initiated at this stage of organ development in the absence of hox11 protein. Thus *hox11* is not required to initiate spleen development but is essential for the survival of splenic precursors during organogenesis. This function for *hox11* suggests that enhanced cell survival may result from the t(10;14) which activates *HOX11* in T cell leukaemias, further strengthening the association between oncogene-induced cell survival and tumorigenesis.

Key words: cancer, homeodomain, translocation, leukaemia, apoptosis, asplenia, *Hox11*, mouse, human

INTRODUCTION

The study of chromosomal translocation breakpoints in human leukaemias has been important for the identification of novel oncogenes involved in the aetiology of the disease (Rabbitts, 1994). T cell acute leukaemia-associated translocations are among the best documented and a number of novel genes have been identified. The cloning of a T cell acute leukaemia translocation t(10;14)(q24;q11) breakpoint identified the *HOX11* gene located at chromosome 10, band q24, which encodes a homeodomain-containing protein (Dube et al., 1991; Hatano et al., 1991; Kennedy et al., 1991; Lu et al., 1991). This nuclear protein has specific DNA-binding and transcriptional transactivation properties (Dear et al., 1993) and is presumed to work by activation of downstream target genes. Like other T cell acute leukaemia-activated proto-oncogenes, *HOX11* does not seem to be expressed in normal T cells (Zutter et al., 1990; Hatano et al., 1991) although one report suggests mature T cells do express HOX11 (Lu et al., 1992).

As homeobox-containing genes are often involved in development (Krumlauf, 1994), *HOX11* could have such a role. We have attempted to assess the function of *hox11* in mouse development using a gene targeting strategy in which the bacterial *lacZ* gene was incorporated into *hox11* by homologous recombination in ES cells. Mice heterozygous for this homologous recombination are normal but express β-galactosidase, under the control of the endogenous *hox11* promoter. Here we show that *hox11* –/– mice are asplenic at birth as previously reported (Roberts et al., 1994) but that spleen formation is initiated in early embryogenesis followed by atrophy of the organ due to apoptosis. Thus, the function of *hox11* during spleen development is in cell survival.

MATERIALS AND METHODS

Preparation of *hox11* targeting clone

Hox11 genomic clones were obtained by screening a λEMBL3 library made using 129-derived CCE ES cell DNA (a gift from Dr G. Grosveld). A 5.3 kb *Bam*HI fragment, encompassing exon 1 of murine *hox11*, was subcloned into pBluescript. An *Sfi*I site was introduced after codon 50 of the predicted *hox11* coding region and a 3.5 kb fragment containing the *E. coli lacZ* gene and SV40 poly(A) signal, generated by PCR from the plasmid pZA (Allen et al., 1988), were inserted into this *Sfi*I site. The predicted coding sequence across the junction is L (amino acid 50 of Hox11)-G-R-S-G-Q-I (amino acid 2 of β-galactosidase). A 2.2 kb fragment encompassing exon 2 and part of exon 3 was amplified by PCR from ES E14 genomic DNA and inserted downstream of the *lacZ* gene. A 1.2 kb pMC1-neo poly(A) fragment (Stratagene, La Jolla, California) containing *Pac*I cohesive

ends was inserted into a *Pac*I site which had been introduced into the intervening sequence between the *lacZ* gene and the 3′ region of *hox11* homology. Three copies of a 1.8 kb pMC1-TK neo *Xho*I cassette (Thomas and Capecchi, 1987) were introduced in tandem into the vector at the end of the right arm of homology to generate the final targeting construct (see Fig. 1A).

Generation and analysis of mutant mice

The ES cell line E14 was maintained as previously described (Robertson, 1987). ES cells (3×10^7) were electroporated with 36.5 μg targeting construct, linearized at a unique *Xho*I site, in 200 μl PBS at 400 V, 25 μF in a Bio-Rad Gene Pulser apparatus as described (Warren et al., 1994). The cells were plated onto confluent feeder layers of mitomycin-C treated neomycin-resistant primary murine embryonic fibroblasts. After 48 hours, selection was initiated by the addition of 400 μg/ml G418 (GIBCO) and 1 μM 5-FIAU {1-(2-deoxy, 2-fluoro-β-D-arabinofuranosil)-5-iodouracil; Bristol-Myers}. Individual colonies were picked 8-11 days after electroporation and cultured on feeder cells in 24-well plates. Clones were frozen and a small number of cells replated into individual wells of a 24-well plate and expanded for isolation of DNA. The enrichment by negative selection with 5-FIAU varied from 9- to 12-fold between transfections.

Clones with a targeted allele were karyotyped using standard procedures (Robertson, 1987) to confirm that they possessed a normal diploid number of chromosomes with no observable gross rearrangements. Chimaeric mice were generated by injection of C57BL/6 blastocysts and were bred with MF1 animals. Offspring that were heterozygous for the deletion were crossed to produce *hox11* null mice. Cell and tail DNAs were prepared as described (Robertson, 1987). DNA was digested with *Bam*HI and examined by Southern blot analysis as previously described (Rabbitts et al., 1993).

Analysis of mouse embryos

Embryos were fixed and staining of whole embryos, or individual tissues, for β-galactosidase activity was as previously described (Allen et al., 1988). No discernible differences were observed between +/− mutant mice and +/+ littermates. For the apoptosis study, cells were dispersed and centrifuged onto microscope slides. Apoptotic cells were detected using the Apoptag *In Situ* Apoptosis Detection Kit (Oncor, Gaithesburg, MD).

For in situ hybridization, mouse embryos were removed, embedded in Tissue Tek (Miles, Elkhart, IN) and 12 μm frozen sections were cut. The procedures for fixation, probe preparation and hybridization are reported elsewhere (Wisden et al., 1987). The oligonucleotide probe for *hox11* in situ hybridization was a 45-mer complementary to the sequence of the 3′ end of exon 1 of *hox11*, 5′-TGA ACC TGT CCT TTG TGT ATC TGC GGT TAC TCT CCA TCC AGG GAA-3′.

For light microscopy analysis, embryos and tissues were embedded in paraffin, sectioned at 4 μm thickness and stained with haematoxylin and eosin.

RESULTS

Null mutations in *hox11* using *lacZ* insertion and examination of β-galactosidase expression in mice

The targeting vector used to produce the null *hox11* mutation had the bacterial *lacZ* gene fused in-frame with codon 50 of *hox11* and the neomycin selectable marker on the 3′ side of this gene (Fig. 1A). The region of homology with the genomic *hox11* was contiguous on the 5′ side of the gene but on the 3′ side a deletion of 1.9Kb was made removing codons 51 to 189 of *hox11* (i.e. the 3′ end of exon 1). Homologous recombinants of *hox11* in E14 or HM1 ES cells were derived using positive-negative selection (Thomas and Capecchi, 1987). The identity of *hox11* targeted clones was established by filter hybridization using a probe from the *hox11* gene just to the 3′ side of the targeting vector (Fig. 1A). A neomycin probe was also hybridized showing that only a single integration event had occurred and a 5′ probe was used to verify the targeting event

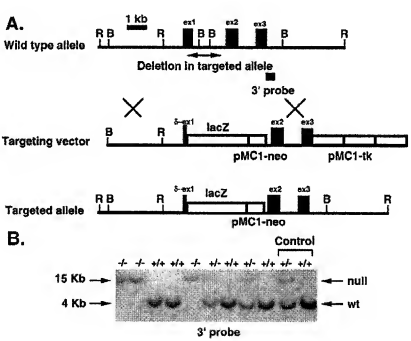

Fig. 1. Gene targeting strategy to incorporate *lacZ* into exon 1 of *hox11* to create a *null/lacZ* allele. (A) A map of the mouse *hox11* gene is shown (wild-type allele), indicating the three exons (indicated as ex1, ex2 and ex3; the homeodomain is in exon 2). The location of a 1.8 kb deletion, including the 3′ end of exon 1 (indicated as δ-ex1), and which is introduced by the gene targeting event, is shown together with the position of the 3′ probe used to detect homologous recombination events. The middle map depicts the targeting vector. The vector was pBlueScript carrying a 7.5 kb genomic *hox11* fragment in which the *lacZ* gene had been cloned in-frame with *hox11* exon 1 and downstream of which was cloned the MC1-neomycin cassette to allow selection of homologous recombinants. Negative selection was facilitated by cloning an HSV thymidine kinase cassette adjacent to the genomic segment. The structure of the *hox11* targeted allele, now with a deleted exon 1 (δ-ex1) containing lacZ and MC1-neo, is depicted at the bottom. R, *Eco* RI; B, *Bam* HI. (B) Southern filter hybridization of DNA isolated from individual mice of a litter resulting from mating between *hox11* +/− parents. DNA was digested to completion with *Bam* HI and hybridized with the 3′ *hox11* probe. The wild-type *hox11* allele is 4 kb while the mutant null allele is 15 kb. This litter, analysed at 6 weeks, contained four +/+, two +/− and three −/− mice. To detect the targeting event, a 300 bp probe was used which encompassed the 3′ end of exon 3 and some flanking DNA.

(data not shown). A number of chimaeric animals were obtained after injection of targeted clones into blastocysts and germline transmission was obtained for two separate clones. Live progeny produced from crosses between heterozygous *hox11* +/– animals were analysed by filter hybridization of tail DNA and the results showed that litters included viable progeny with the *hox11* –/– genotype (Fig. 1B). Thus *hox11* –/– mice can survive to birth. These mice were found to develop apparently normally and to be fertile allowing the establishment of a colony of *hox11* –/– mutant mice.

For analysis of β-galactosidase expression from the targeted *hox11* allele, embryos heterozygous for the null mutation were used. β-galactosidase activity was observed in *hox11* +/– embryos from the earliest time point analysed (E9.5). Expression occurs in several distinct areas, including in the first and second branchial arches (Fig. 2, E9.5) and in the first branchial arch derivatives, there is expression in the epithelial layer of the tongue (Fig. 2, see E13.5). There is also prominent expression in the developing hindbrain along the pontine flexure, which separates the mid-brain from pons and medulla. Staining occurs down the dorsal side of the embryo (Fig. 2, see E11.5) which probably corresponds to cells of the developing spinal cord. Additional expression is observed in the thoracic region in the presumptive pharynx and the outflow tracts of the heart (Fig. 2) and also in the external auditory meatus (Fig. 2, see E12.5 and E13.5). In the gut region from E11.5, in hox11 +/– embryos, there is prominent expression in mesenchymal cells located below the developing stomach which correspond to the area of the developing spleen. With some notable exceptions, such as the heart expression, these results concur with those previously published (Raju et al., 1993; Roberts et al., 1994).

Spleen initiation and atrophy in homozygous null mutant *hox11* mice

The importance of *hox11* expression for development in the

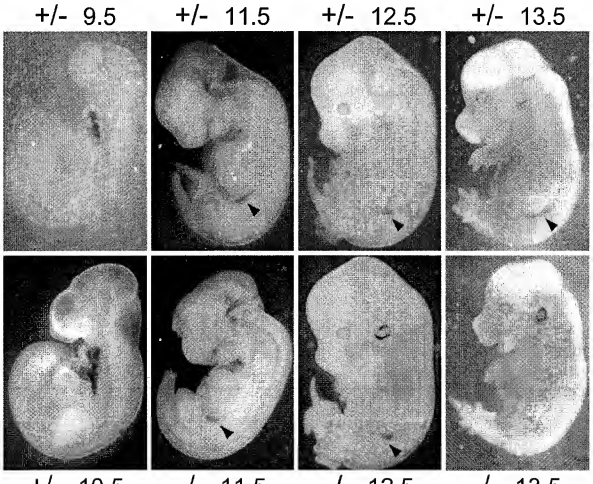

Fig. 2. Homozygous *hox11* null mutant mice exhibit atrophy of the developing spleen. Progeny from matings between *hox11* +/– mice were analysed at different times. Embryos from the different time points were photographed after being fixed and stained for β-galactosidase activity. Genotyping was done either by analysis of yolk sac DNA in the case of E9.5 and 10.5 or by analysis of embryo DNA (subsequent to photography) in the case of E11.5, E12.5 and E13.5. For E9.5 and E10.5, only +/– embryos are shown. Note the presence of spleen at E11.5 and E12.5 but not E13.5 in –/– mice. The arrowhead indicates the developing spleen.

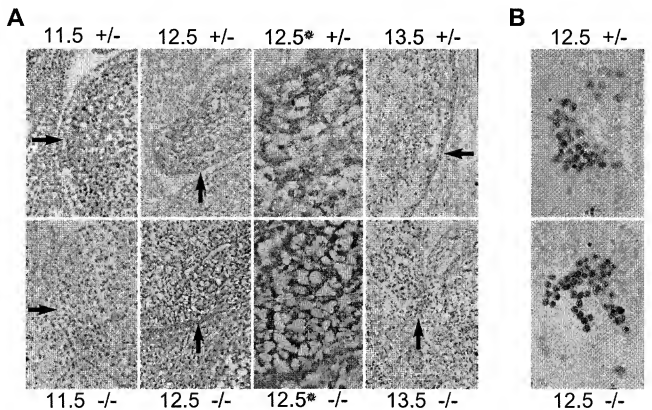

Fig. 3. Histological analysis of the spleen primordium in *hox11* +/– and –/– embryos and apoptosis assay on E12.5 spleen cells. (A) Embryos from different time points were stained for β-galactosidase activity, embedded in paraffin and sectioned sagitally (E11.5) or transversely (E12.5 and E13.5) followed by counterstaining with haematoxylin and eosin (purple/blue). Regions shown correspond to the abdominal area around the developing spleen. The arrow indicates the developing spleen or its remnant. Magnification is 400×, except where denoted by an asterisk, indicating 1000× magnification. (B) Spleens were removed from representative E12.5 *hox11* +/– and –/– embryos. Cells were dispersed, dried onto microscope slides and assayed for presence of free 3′–OH ends by a direct immunoperoxidase method. Brown colour, due to peroxidase staining, denotes the presence of extensive double-strand breaks in DNA indicative of apoptosis. Note that the brown stain is present only in –/– cells. Magnification is 1000×.

areas delineated above was assessed using homozygous null mutant mice. No discernible differences were observed in β-galactosidase patterns between +/– and –/– embryos in any of the above regions of *hox11* expression after E9.5 except with respect to the spleen. Newborn *hox11* –/– mice were examined in detail and found to be asplenic (data not shown) as reported elsewhere (Roberts et al., 1994). However, although live-born animals develop without a spleen, the *hox11* –/– mutant embryos develop a spleen anlage when examined at E11.5 and E12.5 but it is absent after E13.5 (Fig. 2).

During embryogenesis, *hox11* expression in the spleen anlage of +/– embryos is first evident at around E11.5 as assessed by β-galactosidase activity (Fig. 2). In the *hox11* –/– embryos at E11.5 the spleen anlage shows the presence of an apparently normal and healthy population of splenic precursors by histological analysis (Fig. 3A). Furthermore, at E12.5, the splenic anlage persists in –/– embryos (Fig. 2) and the spleen cells retain a healthy appearance (Fig. 3A). In contrast, by E13.5, the spleen anlage was no longer anatomically detectable in *hox11* –/– embryos with only a few residual cells expressing β-galactosidase remaining (Fig. 3A) as compared to a normal developing spleen in *hox11* +/– embryos at E13.5.

The extensive deletion of splenic cells over such a short time in the *hox11* –/– embryos, with no apparent cell lysis or inflammatory response, is suggestive of apoptosis (Kerr et al., 1972; Savill et al., 1993). A characteristic of this type of cell death is the occurrence of multiple internal double-strand DNA breaks within the chromosomal DNA (Wyllie et al., 1984) which can be assayed by labelling these free DNA ends (Wilsman et al., 1993). Using this analysis of *hox 11* +/– and –/– spleen cells, no labelling was observed in E11.5 spleen cells in either +/– or –/– embryos (data not shown). However, at E12.5 a significant proportion of –/– spleen cells were labelled indicative of increased numbers of free chromosomal DNA ends and thus of apoptosis (Fig. 3B) while there was no detectable labelling in corresponding *hox11* +/– spleen cells which are undergoing normal proliferation during spleen organogenesis (Fig. 3B). Therefore the results suggest that a pathway of apoptotic cell death occurs in spleen cells between E12.5 and E13.5 in the absence of *hox11* in –/– null mutant

mice and that this accounts for the lack of a spleen in newborn mice.

Hox11 is expressed in the spleen capsule and trabeculae

The cellular distribution of *hox11* expression in the spleen can account for the biological atrophy of this organ in *hox11* −/− mice. *Hox11* expression continues in the normal spleen up to at least E18.5 and no expression is found in adult spleen. Sections of a *hox11* +/− E18.5 spleen, stained for β-galactosidase expression and counter stained with haematoxylin and eosin, shows that the outer surface of the capsule is a site of *hox11* gene expression (Fig. 4B) and that the intracellular components (mainly erythroid precursors at this stage) are not expressing *hox11*. In addition, the visceral peritoneum which covers the spleen lacks *hox11* expression. This staining pattern faithfully reflects *hox11* transcription since in situ hybridization to *hox11* mRNA in normal E18.5 embryos gave a similar distribution (Fig. 4A). Higher power examination of β-galactosidase expression in the E18.5 spleen (Fig. 4C,D) shows that staining occurs in some cells that penetrate the parenchyma of the spleen. These cells most likely correspond to the trabeculae, which emerge from the inside of the capsule (this is especially clear in sections which are not counter stained, Fig. 4C). The trabeculae consist mainly of myofibroblasts and collagenous fibres. Thus *hox11* is expressed in the spleen capsule and the trabeculae, which together form the framework of the spleen and may function to modify the size of the spleen by contraction. Inability of these cells to survive in the *hox11* −/− mutant mice is apparently the key to the absence of the spleen in the new-born mice.

DISCUSSION

Hox11 is required for maintenance of the developing spleen

The results presented here demonstrate a cellular function of the *hox11* gene in the development of the spleen and also suggest a biochemical function for the gene in cell survival during spleen organogenesis. Mice that lack *hox11* do not develop a functional spleen because the organ fails after the first few days of its development.

The observed asplenia in *hox11* −/− mice after E13.5 is in agreement with a previous report (Roberts et al., 1994). However, our analysis of the very early stages of spleen development shows that the spleen primordium initially forms at the normal time in *hox11* −/− mice but after initial development there is rapid atrophy, between E12.5 and E13.5. Therefore, the *hox11* gene is needed for cell survival during organogen-

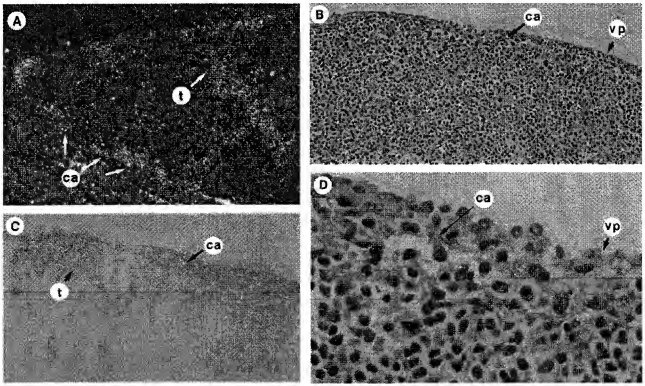

Fig. 4. *Hox11* expression in embryonic day 18.5 spleen. The spleens from E18.5 *hox11* +/+ or +/− mouse embryos were subjected to (A) in situ mRNA hybridization (Wisden et al., 1987) or (B-D) histochemical assay for β-galactosidase activity (Allen et al., 1988). (A) In situ hybridization of a *hox11* antisense oligonucleotide probe with a representative section of a +/+ mouse spleen. (B) A section of a *hox11* +/− spleen stained with X-gal to detect β-galactosidase activity (blue) and counter-stained with haematoxylin and eosin (red/purple). (C) A different section of the same spleen stained only for β-galactosidase. (D) A high power view (1000x) of the section shown in B. Both in situ and histochemical data show *hox11* expression in capsule and trabeculae. ca, capsule; t, trabeculae; vp, visceral peritoneum.

esis of the spleen but not for its initiation. It is also of interest that, although *hox11* is rather specifically expressed in other sites during embryogenesis (see for example Fig. 2), only the survival of spleen cells seems to be affected in the null –/– *hox11* mutant mice. It is possible that this is due to functional redundancy of related genes. At least two other related genes, *hox11L1* and *hox11L2*, are found in the mouse genome (Kennedy et al., 1991; Dear et al., 1993; Raju et al., 1993), and their expression may compensate for the lack of *hox11* expression of *hox11* null mutants.

Our analysis of early spleen development in mutant mice was facilitated by the incorporation of the *lacZ* gene into *hox11*, since the reporter gene acts as a marker, positively identifying cells in embryogenesis which would normally express *hox11* even in –/– mutant embryos. In addition, the developmental fate of cells expressing *hox11* could be compared between +/– and –/– animals. Our detection of initial spleen development and subsequent atrophy in –/– mice differs from a previous report that *hox11* is required for the genesis of the spleen (Roberts et al., 1994). There are several possible explanations for the differences in phenotype. First, the embryos in both experiments are on an outbred genetic background which could lead to alterations in phenotype. Second, the resultant targeted alleles are different in the two reports. However, it is unlikely that a functional protein could occur in the current analysis as most of exon 1 and the flanking intron were deleted during the targeting event.

The cause of the asplenia in the *hox11* –/– mice becomes evident from the specific cellular and developmental pattern of *hox11* expression in the organ. Spleen cells are first visible with β-galactosidase expression at E11.0. Since this coincides with the initial condensation of mesenchymal cells that will form the spleen, *hox11* activation must occur as these cells are organising. In E18.5 +/– spleen, *hox11* expression is confined to the capsule and trabecular structure of the organ. In *hox11* –/– mice, rapid atrophy of the spleen occurs at approximately E12.5 with the organ disappearing within 24 hours. The available evidence argues that a pathway of programmed cell death (apoptosis) is initiated rather than necrosis. For some mammalian cells, it has been suggested that programmed cell death occurs by default unless a 'survival signal' is received (Raff, 1992). Since *HOX11* is presumed to activate downstream target genes, these may function in cell survival in the rather specific biological situation of splenic precursors. Exactly what initiates the death of *hox11* –/– spleen cells is not clear.

A role for *Hox11* in cell survival and T cell tumorigenesis

Our data on the normal function of the Hox11 protein suggests that it plays a role in cell survival. This is presumed to occur via the DNA-binding homeodomain which confers site-specific recognition on the protein (Dear et al., 1993). Thus it is envisaged that target genes are recognised by the protein and either activation or repression of these genes is needed for the function to manifest. Obviously absence of the gene product would influence the expression of these downstream target genes. These arguments suggest a mechanism by which ectopic *HOX11* expression contributes to the tumorigenic phenotype in T cells with translocation t(10;14)(q24;q11) since this, and the related t(7;10)(q35;q24), activate the *HOX11* gene (Boehm et al., 1989; Dube et al., 1991; Hatano et al., 1991; Kennedy et al., 1991; Lu et al., 1991). If *HOX11* functions as a genetic switch that influences genes necessary for cell survival, then its deregulated expression in T cells, after translocation t(10;14) or t(7;10), may enhance the longevity of these specific T cells relative to the unaffected ones. Such longevity may ultimately result in overt tumour occurrence by molecular pathological mechanisms akin to those of the *BCL2* gene (McDonnell et al., 1989; Strasser et al., 1990, 1991; McDonnell and Korsmeyer, 1991; Sentman et al., 1991; Hockenberry et al., 1993; Kane et al., 1993). Activation of *HOX11*, in a subset of T cell leukaemias may therefore be a critical step establishing a T cell clone on the pathway to overt tumour formation.

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